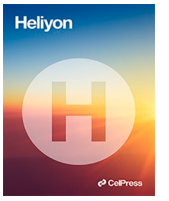
Heliyon 10 (2024) e23223

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



CD163 protein inhibits lipopolysaccharide-induced macrophage transformation from M2 to M1 involved in disruption of the TWEAK–Fn14 interaction

Linjian Chen, Wanchun Mei, Juan Song, Kuncheng Chen, Wei Ni, Lin Wang, Zhaokai Li, Xiaofeng Ge, Liuhang Su, Chenlu Jiang, Binbin Liu *, Cuilian Dai **

Xiamen Cardiovascular Hospital, School of Medicine, Xiamen University, Jinshan Road 2999, Xiamen, 361015, China

ARTICLE INFO

Keywords:
CD163
Macrophage polarization
TWEAK
Fn14
NF-κB
M1

ABSTRACT

Macrophages play a crucial role in regulating inflammation and innate immune responses, and their polarization into distinct phenotypes, such as M1 and M2, is involved in various diseases. However, the specific role of CD163, a scavenger receptor expressed by macrophages, in the transformation of M2 to M1 macrophages remains unclear. Here, dexamethasone-induced M2 macrophages were treated with lipopolysaccharide (LPS) to induce the transformation of M2 to M1 macrophages. We found that treatment with lipopolysaccharide (LPS) induced the transformation of M2-like macrophages to an M1-like phenotype, as evidenced by increased mRNA levels of *Il1b* and *Tnf*, decreased mRNA levels of *Cd206* and *Il10*, and increased TNF-α secretion. Knockdown of CD163 enhanced the phenotypic features of M1 macrophages, while treatment with recombinant CD163 protein (rmCD163) inhibited the LPS-induced M2-to-M1 transformation. Furthermore, LPS stimulation resulted in the activation of P38, ERK, JNK, and NF-κB P65 signaling pathways, and this activation was increased after CD163 knockdown and suppressed after rmCD163 treatment during macrophage transformation. Additionally, we observed that LPS treatment reduced the expression of CD163 in dexamethasone-induced M2 macrophages, leading to a decrease in the CD163-TWEAK complex and an increase in the interaction between TWEAK and Fn14. Overall, our findings suggest that rmCD163 can inhibit the LPS-induced transformation of M2 macrophages to M1 by disrupting the TWEAK-Fn14 interaction and modulating the MAPK–NF–κB pathway.

1. Introduction

Inflammation is a crucial response of the host immune system to harmful stimuli, including pathogens, damaged cells, toxic compounds, and irradiation [1]. Macrophages, widely distributed innate immune cells, play a vital role in mediating the immune response against foreign pathogens and various harmful stimuli such as bacteria, physical stress, and injury [2,3]. Macrophages exhibit heterogeneity and can be classified into two main phenotypes: classical M1 macrophages and alternative M2 macrophages [4]. M1 macrophages express markers such as CD80, CD68, and iNOS, and secrete proinflammatory cytokines like interleukin (IL)-1β and

* Corresponding author. Xiamen Cardiovascular Hospital, School of Medicine, Xiamen University, Jinshan Road 2999, Xiamen, 361015, China.
** Corresponding author.
E-mail addresses: liubinbin@xmu.edu.cn (B. Liu), cuiliand2013@126.com (C. Dai).

https://doi.org/10.1016/j.heliyon.2023.e23223
Received 7 June 2023; Received in revised form 21 November 2023; Accepted 29 November 2023
Available online 4 December 2023

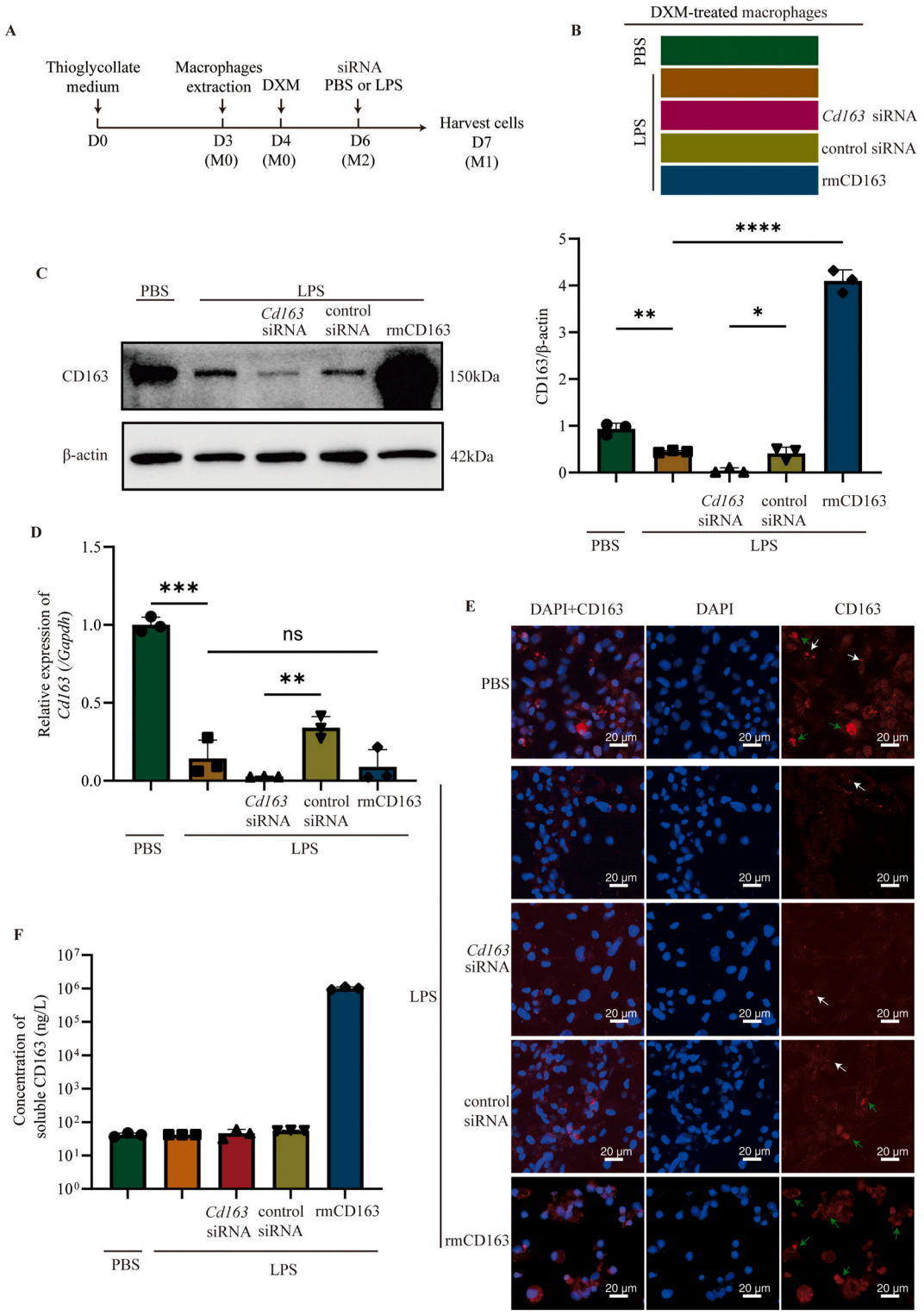


Fig. 1. The status of CD163 during the LPS-induced transformation of macrophages from M2 to M1. (**A**) The experiment procedure of LPS-induced transformation of M2 macrophages to M1. (**B**) The groups of treatment. The protein (**C**) and transcription (**D**) levels of CD163 were measured in macrophages under indicated treatment. (**E**) Immunofluorescent staining of CD163 was performed in macrophages under indicated treatment. The scar bar indicated 20 μm. (**F**) The soluble CD163 levels were measured by ELISA using the superannuates of cell culture media. N = 3.

tumor necrosis factor alpha (TNFα) [5]. In contrast, M2 macrophages express markers such as CD206, CD163, anti-inflammatory cytokines like IL-10, and chemokines like CCL17 [6]. The balance between M1 and M2 macrophages has been associated with the prognosis of various diseases, including autoimmune neuropathy, myocarditis, viral infection, atherosclerosis, sepsis, arthritis, diabetes, and cancer [7,8]. Therefore, unraveling the underlying mechanisms of macrophage transformation from M2 to M1 is crucial for the treatment of these diseases.

CD163 is a scavenger receptor expressed in macrophages and other derived cells [9,10]. Its expression in macrophages is heterogenous, with CD163 being present in red pulp macrophages, Kupffer cells, and perivascular macrophages, but not in perifollicular macrophages and microglia [11]. CD163 expression is particularly high in M2 macrophages, which are associated with an anti-inflammatory phenotype [12]. Treatment of monocytes with glucocorticoids can significantly increase the percentage of CD163-positive monocytes from 10% to 30 % to about 90 % [13] and upregulate CD163 expression levels by more than 40-fold, surpassing the effects of IL-4 treatment [14]. Structurally, CD163 consists of a cytoplasmic tail, a transmembrane segment, and an extracellular domain composed of nine cysteine-rich scavenger receptor class B domains [15]. CD163 plays an anti-inflammatory role by mediating the uptake and clearance of proinflammatory molecules such as hemoglobin [16], TNF-α-like weak inducer of apoptosis (TWEAK) [17], and high-mobility group box 1 protein [18]. It has been reported that lipopolysaccharide (LPS) can induce the transformation of M2 macrophages to M1 in vitro [19] and trigger the shedding of CD163 from the cell surface [20]. However, the specific role of CD163, as well as recombinant CD163 protein (rmCD163), in the transformation of M2 macrophages to M1 remains unclear.

The nuclear factor-κB (NF-κB) signaling pathway, which is activated by MAP kinase (MAPK) cascades, plays a crucial role in immune responses [21,22]. In macrophages, activation of NF-κB leads to the expression of proinflammatory cytokines such as IL-1β and TNF-α [23,24]. Studies have suggested that NF-κB activation contributes to the transformation of M2 macrophages to the M1 phenotype in the tumor microenvironment [25,26]. Additionally, the cytokine TWEAK and its receptor Fn14 have been implicated in NF-κB activation and the induction of proinflammatory cytokines [27,28]. The interaction between TWEAK and CD163 on the cell surface can competitively inhibit the binding of TWEAK to Fn14 [13]. However, it remains unknown whether CD163 can regulate the NF-κB and Fn14 pathway during macrophage transformation.

In this study, we collected dexamethasone (DXM)-induced M2 macrophages from mice and treated them with LPS to investigate the roles of CD163 and recombinant CD163 protein (rmCD163) in the transformation of macrophages from the M2 to the M1 phenotype in vitro. We examined the activation of MAPK-mediated NF-κB signaling and the competitive interactions among CD163, Fn14, and TWEAK during the LPS-induced transformation of M2 to M1 macrophages.

2. Methods

2.1. Cell culture and transfection

Primary intraperitoneal macrophages were collected from male C57BL6/J mice according to previously described methods [29]. The mice were housed in specific pathogen-free facilities at a temperature of 22–23 °C with a 12-h light:12-h dark cycle at Xiamen University. All experimental procedures were performed in accordance with the guidelines outlined by the National Institutes of Health for the Care and Use of Laboratory Animals and were approved by the Animal Care and Protection Committee of Xiamen University (Protocol No. XMULAC20200150). Briefly, macrophages were collected from the peritoneal cavity after three days of stimulation with thioglycolate-containing medium (cat. No. T9032, Sigma, St. Louis, MO, USA). After centrifugation, the macrophages were cultured in Dulbecco's modified Eagle medium (cat. No. 11885084, Gibco, Grand Island, NY, USA) supplemented with 5 % fetal bovine serum (cat. No. 10099141C, Gibco) at 37 °C in a 5 % CO2 environment. After 2–3 h of incubation, the medium was changed to remove the non-adherent cells, and the macrophages were then treated with 1 nM dexamethasone (DXM) (cat. No. ID0170, Solarbio, Beijing, China) for 48 h to induce M2 macrophage polarization (Fig. 1A).

For the transfection of dexamethasone-induced M2 macrophages, 100 nM *Cd163* siRNA or control siRNA (Riobio, Beijing, China) was used along with riboFECT™ CP Reagent (Riobio) following the manufacturer's instructions. Subsequently, the cells were treated with 1 μg/ml lipopolysaccharide (LPS) (cat. No. L8880, Solarbio) for 24 h. The sequence of the *Cd163* siRNA was CTGTGATAATTTCGAAGAA.

In the case of mouse rmCD163 treatment, the cells were treated with 0.5 μg/ml rmCD163 (cat. No. 7435, R&D Systems, Minneapolis, MN, USA) that was dissolved in a 5 mM CaCl2 buffer (cat. No. G0071, Solarbio) for 24 h prior to LPS treatment.

2.2. RNA extraction and quantitative real time polymerase chain reaction (qRT-PCR)

RNA extraction were performed following our previously reported methods [30]. Cells were washed three times with pre-cooled phosphate-buffered saline. TRIzol reagent (cat. No. 390206, Ambion, Naugatuk, CT, USA) was added directly to the cell culture plates for RNA extraction, and RNA was purified through chloroform-isopropyl alcohol precipitation. After washing with 70 % ethanol, RNA was dissolved in nuclease-free water. The concentration and purity of RNA were determined using a NanoDrop micro-volume spectrophotometer (ThermoFisher Scientific, Waltham, MA, USA).

For cDNA synthesis, 500 ng of RNA was reverse transcribed using the cDNA reverse kit with gDNA eraser (cat. No. RR047A, Takara, Tokyo, Japan) according to the manufacturer's instructions. The qRT-PCR was performed using the SYBR mix kit (cat. No. 11204SE08, Yeasen, Beijing, China) on a StepOnePlus PCR system (ThermoFisher Scientific) following the manufacturer's instructions. The PCR conditions consisted of an initial denaturation step at 95 °C for 1 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing

at 60 °C for 20 s, and extension at 72 °C for 40 s, during which the SYBR Green signals were acquired. The threshold cycle (Ct) was used for quantification. The expression levels of *Il1b, Tnf, Il10, Cd206, and Cd163* were normalized to that of *Gadph*. The primer sequences are listed in Table 1.

2.3. Western blotting

Proteins were extracted from macrophages using RIPA buffer (cat. No. R0010, Solarbio) and quantified using the BCA protein assay kit (cat. No. 2225, ThermoFisher Scientific). Western blotting was performed according to previously described methods [30]. Briefly, equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After transferring the proteins onto membranes, the membranes were blocked and incubated with primary antibodies as follows: CD163 (1:1000, cat. No. Ab182422, Abcam, Cambridge, MA, USA), β-actin (1:1000, cat. No. AF5001, Beyotime), tubulin (1:1000, cat. No. AF5012, Beyotime), p-P38 (1:1000, cat. No. 9216 S, Cell Signaling Technology, Danvers, MA, USA), P38 (1:1000, cat. No. 9212 S, Cell Signaling Technology), *p*-ERK (1:1000, cat. No. 9101 S, Cell Signaling Technology), ERK (1:1000, cat. No. 9696 S, Cell Signaling Technology), *p*-JNK (1:1000, cat. No. 9255 S, Cell Signaling Technology), JNK (1:1000, cat. No. 9252 S, Cell Signaling Technology), p-P65 (1:1000, cat. No. AF2006, Affinity Biosciences, OH, USA), P65 (1:1000, cat. No. AF5006, Affinity Biosciences), and TWEAK (1:2000, cat. No. Ab37170, Abcam). Horseradish peroxidase-conjugated anti-mouse (1:10,000, cat. No. 115-035-003, Jackson Laboratories, West Grove, PA, USA) and anti-rabbit (1:10,000, cat. No. 111-035-003, Jackson Laboratories) secondary antibodies were used. The membranes were developed using enhanced chemiluminescent reagents (cat. No. K-12045-D50, Advansta, Menlo Park, CA, USA) and visualized using the ChemiDOC Touch imaging system (BIO-RAD, Hercules, CA, USA). The intensity of each target protein band was quantified using Image J software (National Institutes of Health, Bethesda, MD, USA). The whole gel images are shown in Supplementary Material.

2.4. Co-immunoprecipitation (Co-IP)

Co-IP was performed according to previously described methods [31]. In brief, proteins were extracted from macrophages using NEB buffer (1 % NP40, 150 mM NaCl, 50 mM HCl, pH 8.0) and quantified using the BCA protein assay kit. Approximately 4 mg of protein was used for immunoprecipitation. The proteins were incubated with 2 μg of the CD163 antibody (cat. No. GTX54458, GeneTex, Irvine, CA, USA) or 2 μg of the Fn14 antibody (cat. No. Ab109365, Abcam) overnight at 4 °C. Protein G dynabeads (cat. No. 1004D, ThermoFisher Scientific) were used to collect the protein complexes. After washing with NEB buffer, the proteins were extracted using sodium dodecyl sulfate loading buffer (cat. No. P0015L, Beyotime) and subjected to western blotting analysis.

2.5. Immunofluorescent staining

Cells were fixed with 4 % paraformaldehyde, permeabilized with 0.1 % Triton X-100, and blocked with 3 % BSA in phosphate-buffered saline. The cells were then incubated with the primary antibody against NF-κB p65 (1:200, cat. No. AF5006, Affinity Biosciences) or PE anti-mouse CD163 antibody (156,703, biolegend, San Diego, California, U.S.) overnight at 4 °C. After washing three times, the cells were incubated with a fluorescence-labeled secondary antibody (1:400, cat. No. A11035, ThermoFisher Scientific) and sealed with mounting medium containing 4′,6-diamidino-2-phenylindole (cat. No. P0131, Beyotime, Beijing, China). Images were acquired using an SP5 confocal microscope (Leica, Wetzlar, Germany).

2.6. Enzyme-linked immunosorbent assay (ELISA)

The supernatants of the culture medium were collected from the indicated groups to quantify the concentration of TNF-α using an ELISA kit (MTA00B, R&D Systems, Minneapolis, MN, USA) and IL-10 using an ELISA Kit (RK00016, abclonal, Wuhan, Hubei, China).

Table 1
The sequences of used primers.

Primer	Sequence	Amplicon length
Il1b-Fw	TGAAGTTGACGGACCCCAAA	101bp
Il1b-Rv	TGATGTGCTGCTGCGAGATT	101bp
Tnf-Fw	AAGCCTGTAGCCCACGTCGTA	122bp
Tnf-Rv	GGCACCACTAGTTGGTTGTCTTTG	122bp
Cd206-Fw	TTCGGTGGACTGTGGACGAGCA	108bp
Cd206-Rv	ATAAGCCACCTGCCACTCCGGT	108bp
Il10-Fw	CAGTACAGCCGGGAAGACAATA	151bp
Il10-Rv	GCATTAAGGAGTCGGTTAGCAG	151bp
Cd163-Fw	CTGTGATAATTTCGAAGAAGCCAAAGTTACC	121bp
Cd163-Rv	GGAGCCCCACACGTCTCC	121bp
Gapdh-Fw	TGCACCACCAACTGCTTAG	176bp
Gapdh-Rv	GATGCAGGGATGATGTTC	176bp

2.7. Flow cytometry

Cells were harvested and incubated with FITC-CD206 (141,703, biolegend), PerCP/Cyanine5.5-CD11b (101,228, biolegend), and Brilliant Violet 421-F4/80 (123,132, biolegend) in MACS buffer (comprising 1 × phosphate-buffered saline, 1 % BSA, and 2 mM EDTA)

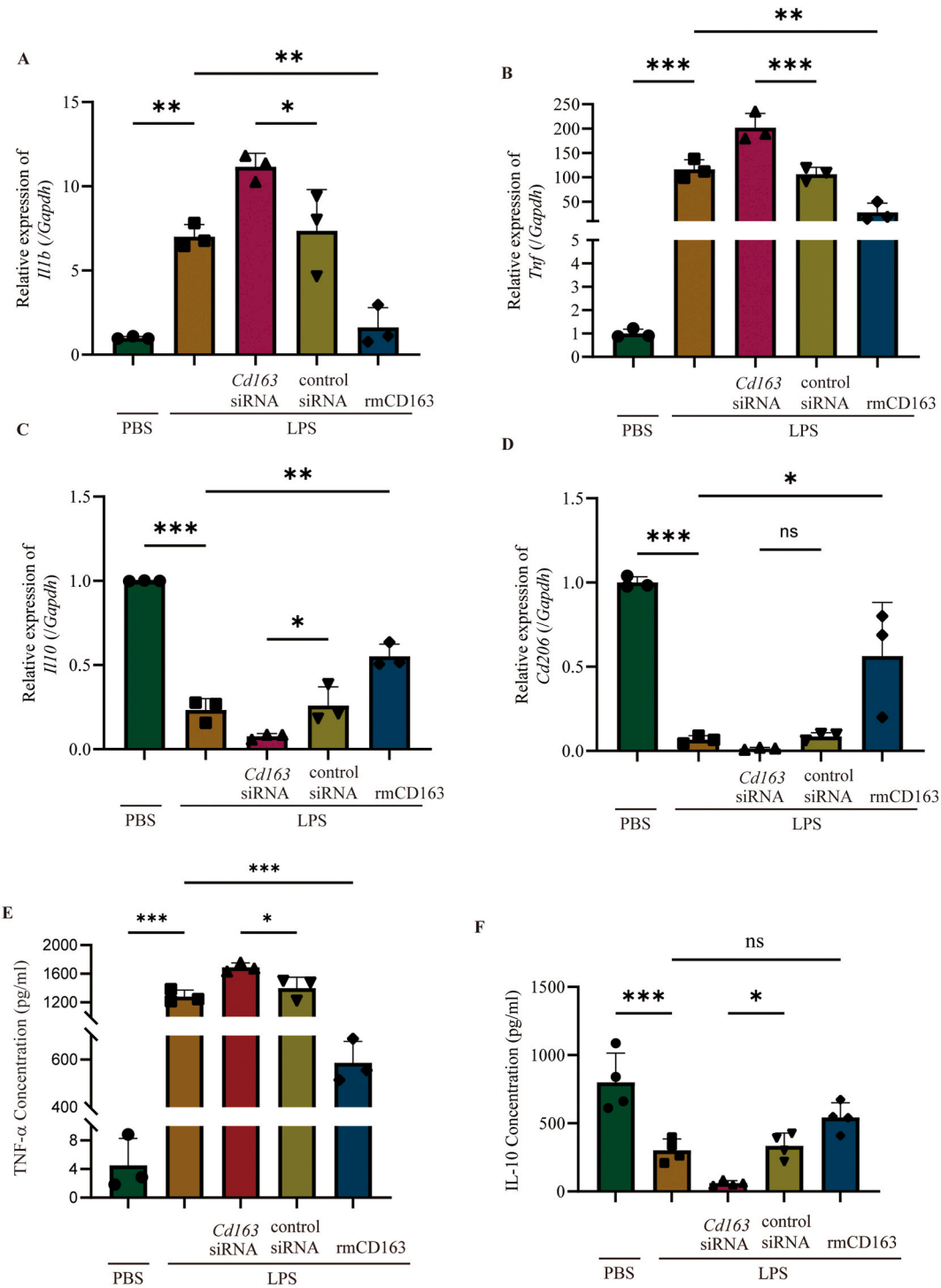


Fig. 2a. CD163 represses the LPS-induced transformation of macrophages from M2 to M1. The relative mRNA levels of *Il1b* (**A**), *Tnf* (**B**), *Il10* (**C**) and *Cd206* (**D**) were quantified by qRT-PCR in macrophages under indicated treatment. (**E**) The concentrations of TNF-α in the culture medium were quantified by ELISA. n = 3. (**F**) The concentrations of IL-10 in the culture medium were quantified by ELISA. n = 4.

for 30 min on ice. Samples were then analyzed using the FACS LSR Fortessa system (Becton Dickinson, Franklin Lakes, USA), and the acquired data were processed using FlowJo software. Doublets or multiplets were excluded from the analysis. Additionally, non-viable cells were identified and excluded based on negative staining with a fixable viability dye (65-0865-14, ThermoFisher Scientific).

2.8. Statistical analysis

Data are presented as mean ± standard deviation (SD). Dots in graphs represent individual cells (n = 3). One-way analysis of variance with Tukey's multiple comparison test was used to analyze the data. *P*-values <0.05 were considered significant. P values are denoted in figures as: not significant (ns), $p > 0.05$; *, $p \leq 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

3. Results

3.1. LPS represses the expression of CD163 during the transformation of M2 to M1 macrophages

Glucocorticoids have been reported to induce the formation of M2 macrophages [32]. Therefore, DXM was used to induce the formation of M2 macrophages, and then lipopolysaccharide (LPS) was used to trigger the transformation of these M2 macrophages into M1 macrophages to understand the role of CD163 during the transformation of M2 to M1 macrophages (Fig. 1A and B). The status of CD163 was analyzed during the LPS-induced transformation of macrophages from M2 to M1. The protein levels (Fig. 1C) and mRNA levels (Fig. 1D) of cellular CD163 were significantly reduced after LPS treatment. CD163 was observed on the cell surface as

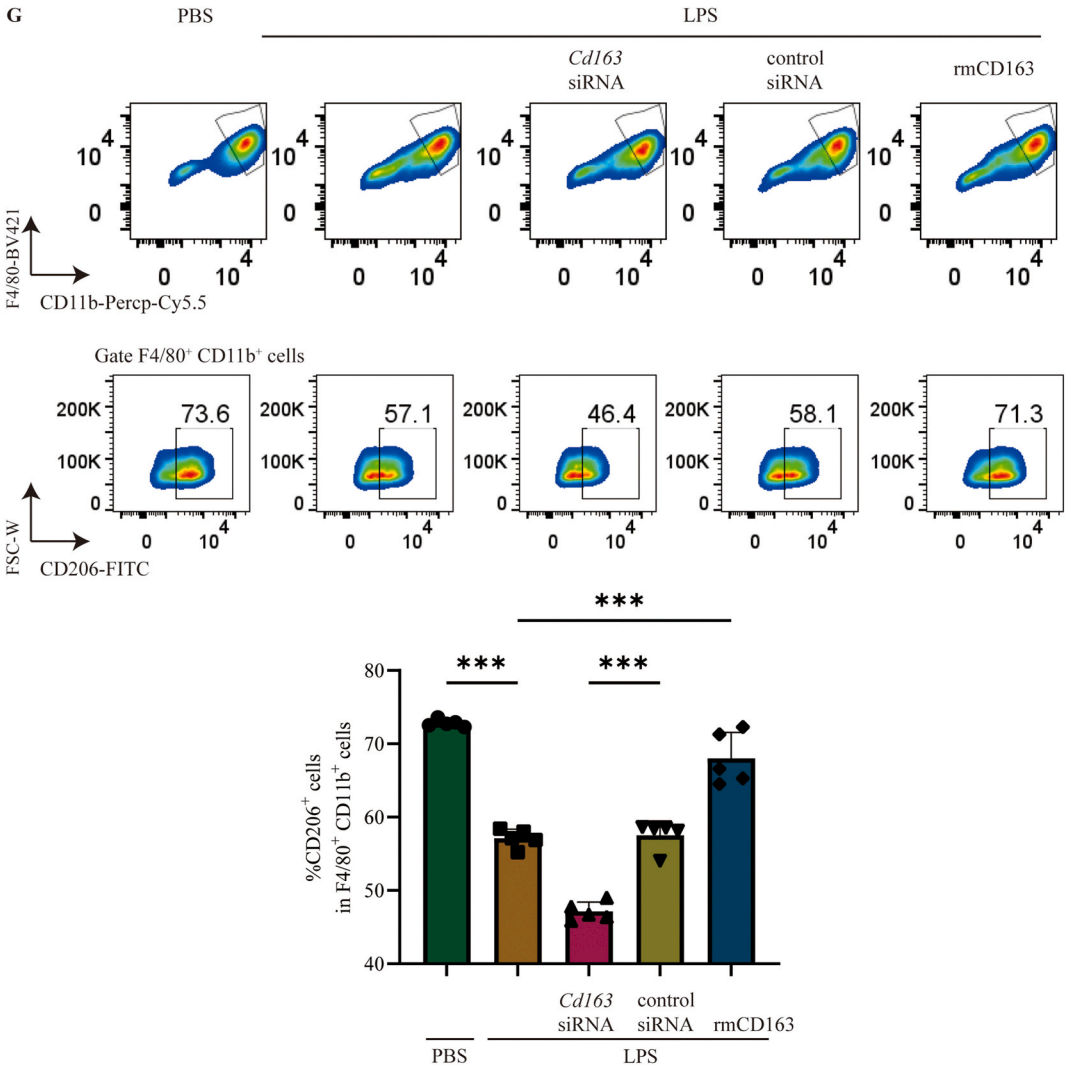


Fig. 2b. CD163 represses the LPS-induced transformation of macrophages from M2 to M1. (**G**) The ratio of $CD206^+$ cells among the $F4/80^+$ $CD11b^+$ macrophage population was determined using flow cytometry. Representative flow cytometry plots are provided. N = 5.

high-brightness dots and near the nucleus as a lot of small dots (Fig. 1E). LPS clearly reduced the fluorescence intensity of CD163, and some low-brightness dots on the cell surface could still be observed (Fig. 1E). The level of soluble CD163 in the medium did not change significantly after LPS addition (Fig. 1F). These findings suggest that LPS reduces the transcription of *Cd163* and does not lead to shedding of CD163 from the cell surface.

Next, we aimed to investigate the role of CD163 in the transformation of M2 macrophages to M1 macrophages by silencing CD163 or adding rmCD163 protein. Initially, the expression of CD163 was effectively reduced in DXM-induced M2 macrophages after siRNA against *Cd163* addition (Fig. 1C–E). During the subsequent LPS-induced transformation from M2 to M1 macrophages, the addition of siRNA against *Cd163* further suppressed cellular CD163 levels compared to the control siRNA (Fig. 1C–E). RmCD163 was added to the cell culture medium, resulting in extremely high levels of cellular CD163 protein (Fig. 1C), intracellular CD163 (Fig. 1E), and soluble CD163 (Fig. 1F) without alteration of the transcription levels (Fig. 1D), indicating that macrophages might scavenge CD163 proteins.

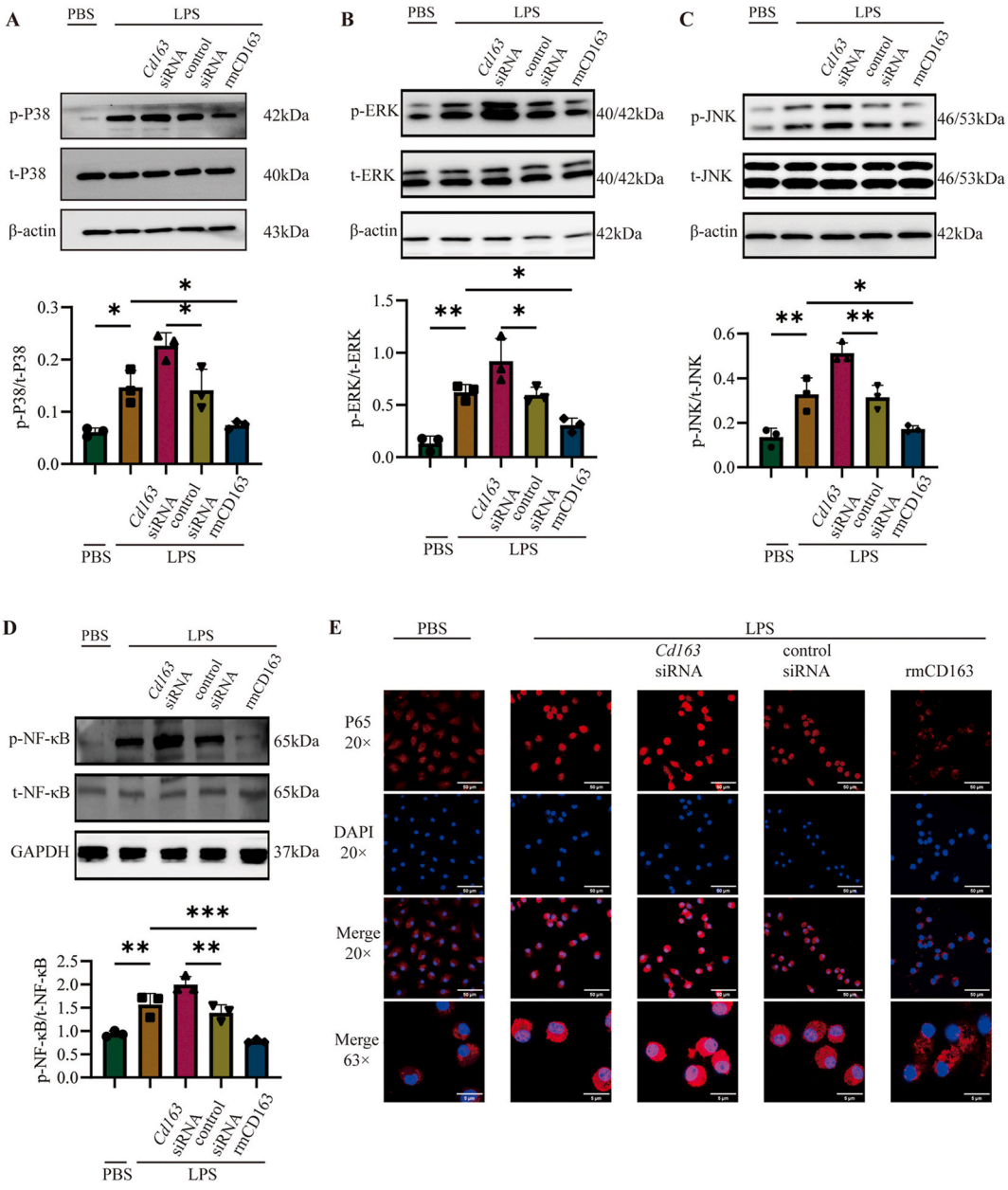


Fig. 3. CD163 represses the LPS-induced activation of the MAPK–NF-κB pathway during the transformation of macrophages from M2 to M1. The protein levels of phosphorylated and total P38 (**A**), ERK (**B**), JNK (**C**), and P65 (**D**) were quantified by western blotting in macrophages as indicated treatment. (**E**) Immunofluorescent staining of P65 was performed in macrophages as indicated treatment. The scar bar indicated 50 μm. N = 3.

3.2. CD163 represses the LPS-induced transformation of M2 to M1 macrophages

Further, the mRNA levels of *Il1b* (Fig. 2a) and *Tnf* (Fig. 2b), which are specific markers of M1 macrophages, increased, while the mRNA levels of *Il10* (Fig. 2c) and *Cd206* (Fig. 2d), which are specific markers of M2 macrophages, decreased upon LPS treatment. This indicates that LPS can indeed induce the transformation of DXM-induced M2 macrophages to M1 macrophages, representing a shift from an anti-inflammatory to a pro-inflammatory phenotype. We also observed that LPS further significantly upregulated the mRNA levels of *Il1b* and *Tnf* and downregulated the expression of *Il10* after silencing the expression of CD163. Conversely, treatment with rmCD163 resulted in significant decreases in *Il1b* and *Tnf* expressions and increases in *Il10* and *Cd206* (Fig. 2a–d). The secreted TNF-α concentration, determined by ELISA, indicated that LPS increased the concentration from 4.5 pg/ml to 1281 pg/ml, while siRNA against *Cd163* or rmCD163 significantly increased or decreased the concentration of TNF-α, respectively (Fig. 2e). Conversely, the reduction in IL-10 secretion caused by LPS was further diminished upon the addition of siRNA against *Cd163* (Fig. 2f). The proportion of $CD206^{+}$ cells within macrophages, which represents the percentage of M2 macrophages, was notably decreased after a 24-h treatment with LPS. Additionally, siRNA targeting *Cd163* further exacerbated this decline, while rmCD163 significantly counteracted the LPS-induced reduction (Fig. 2g). These findings suggest that CD163 can repress the LPS-induced transformation of macrophages from M2 to M1. Furthermore, the decreased levels of CD163 induced by LPS may promote the transformation of macrophages

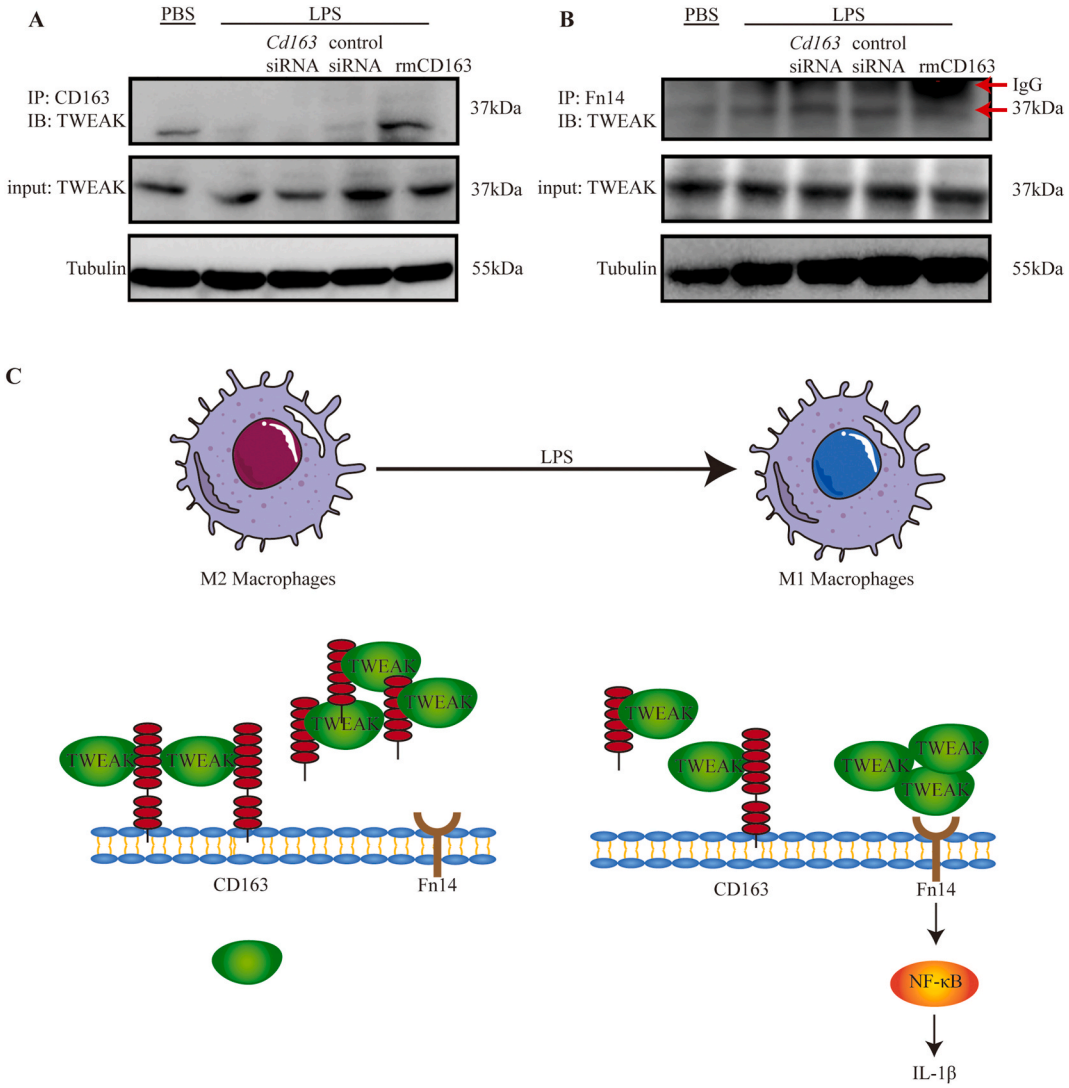


Fig. 4. CD163 blocks the interaction of TWEAK and Fn14 during the LPS-induced transformation of macrophages from M2 to M1. Co-immunoprecipitation was performed to understand the interaction between CD163 and TWEAK (**A**) and the interaction between Fn14 and TWEAK (**B**) in macrophages under indicated treatment. (**C**) The decreased CD163 protein on the surface of macrophages induced by LPS weakened the interaction between TWEAK and CD163 and strengthened the binding of TWEAK and Fn14, which activated the MAPK–NF-κB pathway and induced the transformation of macrophages from M2 to M1.

from M2 to M1, and the addition of rmCD163 may have therapeutic benefits in inhibiting macrophage transformation and suppressing inflammatory responses.

3.3. CD163 represses the LPS-induced activation of the MAPK–nf-κb pathway during the transformation of M2 to M1 macrophages

The MAPK–NF-κB pathway regulates the LPS-induced proinflammatory response and the polarization of macrophages [33]. Thus, western blotting was used to investigate the effect of CD163 on the activation of the MAPK–NF-κB pathway. LPS significantly increased the phosphorylation levels of P38, ERK, JNK and NF-κB P65 in DXM-induced M2 macrophages (Fig. 3A–D). Knockdown of CD163 further enhanced the protein levels of p-P38, *p*-ERK, *p*-JNK, and p-P65 compared to control siRNA-treated cells. The addition of rmCD163 significantly suppressed the LPS-induced phosphorylation of these proteins, indicating that CD163 can regulate the activation of the MAPK–NF–κB pathway during macrophage transformation (Fig. 3A–D). Immunofluorescence staining revealed that in DXM-induced M2 macrophages, NF-κB P65 was predominantly located in the cytoplasm. LPS treatment reduced the cytoplasmic signal and increased the nuclear signal, indicating nuclear translocation of P65 (Fig. 3E). In addition, *Cd163* knockdown resulted in increased staining intensity of P65 in the nucleus compared to cells treated with control siRNA. Conversely, rmCD163 treatment significantly reduced the nuclear signal of P65 (Fig. 3E), suggesting that CD163 can repress the LPS-induced activation of the MAPK–NF–κB pathway, and rmCD163 can repress the LPS-induced activation of the MAPK–NF–κB pathway during macrophage transformation.

3.4. CD163 blocks TWEAK–Fn14 interactions during the LPS-induced transformation of M2 macrophages to M1

To investigate the interactions between CD163, TWEAK, and Fn14 during the LPS-induced transformation of M2 to M1 macrophages, Co-IP experiments were performed. The results showed that the CD163 and TWEAK complex decreased, while the complex between TWEAK and Fn14 increased after LPS induction (Fig. 4A and B). Furthermore, *Cd163* knockdown strengthened the interaction between TWEAK and Fn14 during the LPS-induced transformation of macrophages from M2 to M1, suggesting that CD163 can competitively inhibit the interaction between TWEAK and Fn14 during the LPS-induced transformation of M2 macrophages to M1.

Additionally, rmCD163 was found to bind to TWEAK (Fig. 4A) and repress the binding of TWEAK to Fn14 (Fig. 4B) during the LPS-induced transformation of M2 macrophages to M1, indicating that *rmCD163* competitively blocks the interaction between TWEAK and Fn14.

4. Discussion

In this study, the decreased expression of CD163 was associated with the LPS-induced transformation of M2 macrophages to M1, and soluble CD163 protein could repress the transformation of M2 macrophages to M1. Previous research has also shown that decreased CD163 expression promotes the shift of macrophages towards the M1 phenotype in certain conditions like symptomatic apical periodontitis [34]. Conversely, increased CD163 expression inhibits the M1 macrophage phenotype induced by LPS in human macrophages [35]. Therefore, the downregulation of CD163 expression is a crucial factor in the transformation of M2 macrophages to M1.

Additionally, increased levels of circulating soluble CD163 have been considered as a specific marker of macrophage activation [36]. LPS and phorbol 12-myristate 13-acetate have been reported to induce the shedding of CD163 in human macrophages [37,38]. However, the functions of circulating soluble CD163 are not fully understood, and whether the increased levels of soluble CD163 are the cause or result of various diseases is still largely unknown. In this study, increased soluble CD163 levels were not observed in mouse macrophages after LPS treatment, as mouse CD163 lacks the Arg-Ser-Ser-Arg domain that is responsible for CD163 cleavage by the inflammation-responsive protease ADAM17, leading to the generation of soluble CD163 [39]. Nonetheless, soluble CD163 still demonstrated its ability to repress the transformation of murine macrophages from M2 to M1. Consistently, soluble CD163 has been reported to inhibit inflammation and promote muscle regeneration during tissue ischemia [40]. Therefore, soluble CD163 may play a crucial role in tissue microenvironments to suppress the transformation of M2 macrophages to M1.

One limitation of this study is the absence of *in vivo* assays to gain a deeper understanding of the biological significance of CD163 in the transformation from M2 to M1 macrophages, as the ratio of M1 and M2 macrophages in the tissues has been associated with the prognosis of various diseases [7]. Therefore, further investigations are needed to explore the role of CD163 in the transformation of M2 macrophages to M1 in different tissue microenvironments. Additionally, the classification of macrophages into M1 and M2 phenotypes is a simplified operational concept, and tissue macrophages are heterogeneous cell populations. They are clustered according the expression of *C*–C chemokine receptor type 2 (CCR2) into resident $CCR2^{-}$ and recruited $CCR2^{+}$ subsets originating from yolk sac-derived erythromyeloid progenitors and circulating monocytes, respectively [41]. CD163 is expressed in resident tissue macrophages of adipose tissues [42] and the heart [43]. Adhering to the M1-M2 paradigm, the tissue-resident macrophages are classified as 'M2-like', with fundamental roles in tissue homeostasis that relate to the role of macrophages during development, maintenance of homeostasis and resolution of inflammation [44]. Several reports have also suggested that CD163 can inhibit atherosclerosis [40], repair ischemic tissues [45], arthritis [46], and diabetes [47]. Herein, CD163 recombinant protein was demonstrated to repress the LPS-induced transformation of macrophages from M2 to M1, suggesting that recombinant CD163 may have potential therapeutic benefits in some diseases [40,48]. However, direct evidence supporting these therapeutic benefits is still lacking and further research is needed.

The MAPK–NF–κB pathway was found to be activated during the transformation process, which was repressed by soluble CD163. Previous studies have reported that *S. frutescens* extracts can reduce the ratio of M1 macrophages by inhibiting the MAPK–NF–κB

pathway in RAW 264.7 murine macrophages [49]. Additionally, the negative regulator of NF-κB activation, p50, can inhibit M1 macrophage polarization and promote M2-driven anti-inflammatory responses [50]. Therefore, CD163, as a typical marker of M2 macrophages [51] may control the activation of NF-κB pathway to regulate the polarization of macrophages.

The endosomal-lysosomal system is a series of organelles involved in the endocytic pathway, responsible for internalizing, recycling, and modulating various cargo molecules [52]. CD163 has been implicated in modulating the internalization signal, and it has been observed that the CD163 short tail variant Y1091A mutant inhibits macrophage internalization [53]. In this study, rmCD163 was taken up by macrophages and localized in the perinuclear region (Fig. 2c), which might be perinuclear lysosomes. This suggests that rmCD163 undergoes internalization and trafficking to the lysosomal compartment for potential degradation or further processing. Additionally, CD163 has been reported to mediate the uptake of TWEAK, leading to the repression of Fn14 activation and the subsequent proinflammatory response [17]. The binding of TWEAK to Fn14 activates classical and non-classical cascades within the NF-κB pathway [54,55]. Furthermore, the addition of soluble CD163 protein was found to increase the formation of the CD163-TWEAK complex, which may be internalized by macrophages to induce the degradation of TWEAK. This process inhibits the interaction between TWEAK and Fn14, resulting in the suppression of NF-κB activation and the transformation from an M2 to an M1 phenotype (Fig. 4C).

In summary, CD163 could repress the LPS-induced transformation of M2 to M1 macrophages, which was related to inhibit the activation of the MAPK–NF-κB pathway and the interaction between TWEAK and Fn14 in macrophages (Fig. 3C). And CD163 protein addition may exert a therapeutic effect to inhibit the transformation of M2 macrophages to M1.

Ethics statement

Animal experiments were conducted following the guidelines of the Animal Care and Use Committee at Xiamen University (Permit No. XMULAC20200150).

Funding

The work was supported by grants from the National Natural Science Foundation of China (grant no. 82000304) to Liu B; the National Key Research and Development Program of China (2019YFE0113900), the Xiamen Medical and Health Key Project (3502Z20204005) and the Fujian Provincial Health Technology Project (2021QNB021) to C. Dai.

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Linjian Chen: Conceptualization, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. **Wanchun Mei:** Formal analysis, Investigation. **Juan Song:** Formal analysis, Investigation. **Kuncheng Chen:** Formal analysis, Investigation. **Wei Ni:** Formal analysis, Investigation. **Lin Wang:** Formal analysis, Investigation. **Zhaokai Li:** Data curation, Investigation, Resources. **Xiaofeng Ge:** Data curation, Investigation, Resources. **Liuhang Su:** Data curation, Investigation, Resources. **Chenlu Jiang:** Data curation, Investigation, Resources. **Binbin Liu:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing. **Cuilian Dai:** Conceptualization, Formal analysis, Funding acquisition, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23223.

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